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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 5 : G01N 33/569 | A1 | (11) International Publication Number: WO 94/16331 |
|---|---|---|
| | | (43) International Publication Date: 21 July 1994 (21.07.94) |

(21) International Application Number: PCT/US94/00324

(22) International Filing Date: 10 January 1994 (10.01.94)

(30) Priority Data:
08/006,676 15 January 1993 (15.01.93) US

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(81) Designated States: BR, SD, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*With international search report.*

(54) Title: DIAGNOSIS OF LEISHMANIASIS

(57) Abstract

There is disclosed a method for diagnosing leishmaniasis comprising: (a) obtaining a sample from a patient suspected of being infected with a *Leishmania* parasite, wherein the sample contains antibodies from the patient; and (b) determining the presence of antibodies that bind to a K39 repeat unit antigen from the sample. There is further disclosed an antigen common to many species of *Leishmania* that can be used as a diagnostic agent.

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# DIAGNOSIS OF LEISHMANIASIS

## Technical Field of the Invention

The present invention provides a method for diagnosing leishmaniasis in a patient suspected of being infected with the parasitic protozoa *Leishmania*. The present invention further provides a diagnostic kit for use in diagnosing *Leishmania*, and an antigen useful for diagnosing the presence of *Leishmania* and as a vaccine to prevent infection with *Leishmania*.

## Background of the Invention

The transmission of pathogenic *Leishmania* involves an injection of extracellular promastigotes into a mammalian host by an infected sandfly. The promastigotes rapidly attach and enter monocytes and cells of the reticuloendothelial system, where they transform into amastigotes and multiply within phagolysosomes. Analysis of the interaction of *Leishmania* promastigotes with the target host cell suggests that both parasite and host molecules are involved in cell adhesion. Clinical symptoms of leishmaniasis range from self-healing skin lesions to diffuse cutaneous and mucosal manifestations, or severe visceral involvement of the spleen, liver and lymph nodes (visceral leishmaniasis or VL).

Visceral leishmaniasis is generally caused by *Leishmania donovani* in Africa and India, *L. infantum* in Southern Europe or *L. chagasi* in Latin America. In VL, high levels of parasite specific antibodies are observed prior to detection of antigen specific T cell responses (Ghose et al., *Clin. Exp. Immunol.* 40:318-326, 1980). This antibody response has been exploited for serodiagnosis of infection with *L. chagasi* and *L. donovani*. The current World Health Organization's estimate of 12 million cases of leishmaniasis and recent epidemics of visceral leishmaniasis in Sudan and India highlight the need for more effective early diagnosis and therapeutic agents. Also at least 400,000 new cases of VL are diagnosed annually. The current diagnostic tests to measure an antibody response use whole or lysed parasites. Therefore, there is a need in the art to improve the diagnostic accuracy for diagnosing VL early while the potentially fatal disease is more treatable.

Recovery from leishmaniasis correlates with the development of specific T lymphocyte responses and usually confers long-lasting immunity against reinfection (Carvalho et al., *J. Clin. Invest.* 76:2066-6, 1985 and Carvalho et al., *J. Immunol.* 135:4144-8, 1985). Both recovery from disease and resistance to reinfection are dependent on the development of specific T lymphocyte responses. Interferon gamma (IFN-γ) is a product of activated T cells, has demonstrated anti-leishmania activity *in*

*vitro* (Murray et al., *J. Clin. Invest.* 72:1506, 1983 and Nacy et al., *J. Immunol.* 135:1305, 1985), and *in vivo* (Reed et al., *J. Immunol.* 132:3116, 1984 and Murray et al., *J. Immunol.* 1348:2290, 1987) and has been used effectively in the clinical treatment of leishmaniasis (Harms et al., *Lancet* 10:1287, 1989 and Badaro et al., *N. Engl. J. Med.* 322:16, 1990).

One antigen, called gp63, has been cloned (Miller et al., *Mol. Biochem. Parasitol.* 38, 267-274, 1990) and was found to be a metalloprotease and is highly conserved among different species of *Leishmania* (Etges et al., *J. Biol. Chem.* 261:9098, 1986; Chaudhuri et al., *Mol. Biochem. Parasitol.* 27:43, 1988; Chaudhuri et al., *J. Biol. Chem.* 264:7483, 1989; Colmer-Gould et al., *J. Exp. Med.* 162:902, 1985; and Button et al., *J. Exp. Med.* 167:724, 1988). Gp63 is relatively abundant on both the infective promastigote stage and the intracellular amastigote stage (Frommel et al., *Mol. Biochem. Parasitol.* 38:25-32, 1990 and Medina-Acosta et al., *Mol. Biochem. Parasitol.* 37:263, 1989). Gp63 is important for both parasite entry into macrophages (Russel and Wilheim, *J. Immunol.* 136:2613, 1986; Chang et al., *Proc. Natl. Acad. Sci. USA* 83:100, 1986; Wilson and Hardin, *J. Immunol.* 141:265, 1988; and Mosser and Edelson *J. Immunol.* 135:2785, 1985) and subsequent survival within the phagosome (Chaudhuri et al., *J. Biol. Chem.* 264:7483, 1989). Immunization with native gp63 *in vivo* partially protected susceptible mice against cutaneous disease (Handman and Mitchell *Proc. Natl. Acad. Sci. USA* 82:5910, 1985 and Russel and Alexander *J. Immunol.* 140:1274, 1988). Moreover, recombinant gp63 expressed in *Salmonella* conferred partial protection by oral immunization against *Leishmania major* infection in resistant mice (Yang et al., *J. Immunol.* 145:2281, 1990). Both native gp63 and recombinant gp63 elicited strong proliferative responses, as well as IFN-γ production, from leishmaniasis patients with a spectrum of clinical disease (Russo et al., *J. Immunol.* 147:3575, 1991).

## Summary of the Invention

The present invention provides a method for diagnosing leishmaniasis comprising: (a) obtaining a sample from a patient suspected of being infected with a *Leishmania* parasite, wherein the sample contains antibodies from the patient; and (b) determining the presence of antibodies that bind to a K39 repeat unit antigen from the sample. Preferably the inventive method is a serodiagnostic method utilizing sera from the individual suspected of harboring a *Leishmania* parasite. Preferably the antigen used is one or a plurality of K39 repeat sequences, wherein the K39 repeat sequence comprises the amino acid sequence (in single letter designation) L E Q Q L R (D/E) S E (E/A) R A A E L A S Q L E (A/S) T (A/T) A A K (M/S) S A E Q D R E (N/S) T R A (T/A) or (in three letter designation) Leu Glu Gln Gln Leu Arg (Asp/Glu) Ser Glu

(Glu/Ala) Arg Ala Ala Glu Leu Ala Ser Gln Leu Glu (Ala/Ser) Thr (Ala/Thr) Ala Ala Lys (Met/Ser) Ser Ala Glu Gln Asp Arg Glu (Asn/Ser) Thr Arg Ala (Thr/Ala) (SEQ ID NO:3). Preferably, the inventive method further comprises the step of determining the presence of antibodies that bind to a native or recombinant gp63 polypeptide.

The present invention further provides a diagnostic kit for evaluating a patient antibody-containing sample for the presence of anti-*Leishmania* parasite antibodies, comprising a K39 repeat unit antigen. Preferably, the K39 repeat unit antigen is bound to a solid phase. Preferably, the diagnostic kit further comprises an anti-human antibody conjugated to a detection moiety. Preferably the antigen used is one or a plurality of K39 repeat sequences, wherein the K39 repeat sequence comprises the amino acid sequence Leu Glu Gln Gln Leu Arg (Asp/Glu) Ser Glu (Glu/Ala) Arg Ala Ala Glu Leu Ala Ser Gln Leu Glu (Ala/Ser) Thr (Ala/Thr) Ala Ala Lys (Met/Ser) Ser Ala Glu Gln Asp Arg Glu (Asn/Ser) Thr Arg Ala (Thr/Ala) (SEQ ID NO:3). Most preferably, the diagnostic kit further comprises a gp63 polypeptide, in combination with a K39 repeat unit antigen.

**Brief Description of the Drawings and Sequences**

Figure 1 shows the expression and purification of recombinant K39 (rK39) antigen. The gels shows Coomassie blue-stained 12% SDS-polyacrylamide gel of molecular weight markers (lane 1), *E. coli* lysates from uninduced cells (lane 2), and induced cultures (lane 3) of clone K39, and purified rK39 (lane 4, 2 μg).

Figure 2 shows reactivity of patient sera with rK39. Blots containing *L. chagasi* promastigote lysate (lane 1, 10μg), purified rK39 (lane 2, 50 ng) and *T. cruzi* epimastigote lysate (lane 3, 10 μg) were probed with individual *L. chagasi* VL sera (A-C), individual *L. donovani* VL sera (D-F), or pooled mucosal leishmaniasis (G, n=4), cutaneous leishmaniasis (H, n-4), or *T. cruzi* infection (I, n=5) sera. Pooled normal human sera (n=3) and no primary antibody controls are shown (J and K, respectively). Bound antibody was detected with $^{125}$I-protein A.

Figure 3 shows a Southern blot analysis of the LcKin gene sequences. Genomic DNA (2.5 μg/lane) from *L. chagasi* digested with *Bam* HI (lane 1), *Hind* III (lane 2) and *Pst* I (lane 3) or *Pst* I digested DNA from *L. amazonesis* (lane 4), *L. braziliensis* (lane 5), *L. guyanesis* (lane 6), *L. donovani* (lane 7), *L. infantum* (lane 8), *L. major* (lane 9), or *T. cruzi* (lane 10) were analyzed by Southern blotting. The blots were probed (A) with a 2.4 kb *Hind* III fragment from the LcKin homology domain or (B) with the 915 bp repetitive insert of K39.

Figure 4 shows reactivity of rabbit anti-rK39 antiserum on recombinant and native leishmania lysates. A is an immunoblot of purified rK39 (50 ng per lane) transferred from 12% SDS-polyacrylamide gels and probed with preimmune rabbit

serum (lane 1) or rabbit anti-rK39 (lane 2). B is an immunoblot of *L. chagasi* promastigote (lanes 1 and 5, 10 μg) and amastigote (lanes 2 and 6 10 μg) lysates or *L. amazonensis* promastigote (lanes 3 and 7, 10 μg) and amastigote (lanes 4 and 8, 10 μg) lysates, transferred from 7.5% SDS-polyacrylamide gels and probed with preimmune rabbit serum (lanes 1-4) or rabbit anti-rK39 (lanes 5-8). C is an immunoblot showing reactivity of rabbit antisera raised against *L. chagasi* ribosomal protein PO, described in Skeiky et al. (*J. Exp. Med.* 176:201, 1992), with lanes 1-4 of B.

Figure 5 shows an ELISA evaluation of patient seroreactivity on *L. chagasi* promastigote lysate (panel A) or purified rK39 (panel B). Absorbance values (mean + SEM) of Brazilian VL (VL-B, n=57), Sudanese VL (VL-S, n=52), *T. cruzi* infection (Tc, n=35), Brazilian cutaneous leishmaniasis (CL-B, n=13), Sudanese cutaneous leishmaniasis (CL-S, n=13) mucosal leishmaniasis (ML, n=15) and normal (n=15) sera.

Figure 6 shows patient sera reactivities against recombinant gp63. All sera samples were diluted 1:100 and assayed by an ELISA technique. Individual absorbance values are represented by dots; horizontal and vertical bars represent the mean +/- 95% confidence limit (Student's t-test), respectively. The abbreviations are visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucosal leishmaniasis (MCL).

SEQ ID NO: 1 is the amino acid sequence for K39.

SEQ ID NO: 2 is the DNA sequence encoding a K39 polypeptide.

SEQ ID NO:3 is the amino acid sequence of a 39 amino acid repeat unit antigen.

**Detailed Description of the Invention**

The present invention provides a method for diagnosing VL and a diagnostic kit for VL. The present invention was made possible by the discovery of a K39 gene and its DNA sequence and deduced amino acid sequence having a plurality of antigenic 39 amino acid repeat units. The K39 gene was found in an attempt to characterize leishmania antigens recognized by a spectrum of VL patients, including VL patients infected with either *L. donovani* or *L. chagasi*.

A *L. chagasi* genomic DNA expression library was screened with sera obtained from a patient having VL caused by *L. donovani*. From approximately 32,000 recombinants screened, seven clones were selected based upon reactivity with this patient's sera. The seven clones contained inserts ranging from 0.9 kb to 2.6 kb and expressed immunoreactive recombinant proteins of 35 kDa to 100 kDa. Clone K39 was exceptionally reactive with the test serum. Recombinant K39 antigen (rK39) migrated on Coomassie-stained SDS-PAGE as a 39 kDa protein in induced bacterial

extracts (Figure 1, lane 3). The protein was purified by ammonium sulfate fractionation and preparative isoelectric focusing (Figure 1, lane 4) with a yield of 25-30 mg per liter.

The DNA and deduced amino acid sequences of the insert of clone K39 were determined and are provided in SEQ ID NOs: 1 and 2 herein. The DNA sequence contained a single open reading frame encoding 298 amino acids with a predicted molecular weight of 32.7 kDa and a pI of 4.4. Clone K39 contains an additional 6.2 kDa of plasmid fusion sequences. In this sequence was noted 6.5 copies of a randomly arrayed 39 amino acid repeat sequence. SEQ ID NO: 3 shows the consensus sequence of the repeat unit.

To further characterize this gene, clones containing sequences flanking the K39 gene fragment were isolated from the *L. chagasi* library using a K39 insert probe. Sequence analysis of one overlapping clone, LcKin, showed that the open reading frame extended for 1971 base pairs in the 5' direction, encoding 657 nonrepeditive amino acids. 5' to the putative ATG initiation codon, 454 base pairs of sequence were obtained with multiple termination codons in each reading frame. Partial characterization of clones containing 3' flanking sequences indicated that the repeat domain extended for approximately 3 to 4 kb.

GenPept and Swiss-Protein data bank searches revealed similarity between LcKin and several members of the superfamily of kinesin-related proteins, particularly in the N-terminal motor domain. A relatively high level of sequence conservation was observed in the putative ATP and microtubule binding domains (Yang et al., *Cell* 56:879, 1989). The remaining 500 residues showed little similarity to sequence of the tail regions of kinesin and myosin of a number of species. Secondary structure analysis predicted that this portion of LcKin (amino acids 426-955) contain greater than 90% helical structure, a feature characteristic of a coil-coiled tail regions of several motor proteins. Therefore, the repetitive epitope of the rK39 antigen appears to be present in *L. chagasi* as part of the tail region of a leishmania kinesin-related protein.

The present invention provides an identification of a 230 kDa antigen of *L. chagasi*, LcKin, with sequence homology to the kinesin superfamily of motor proteins. The gene is predominantly expressed by tissue amastigotes. The DNA sequence is present in at least seven diverse species of *Leishmania*. The DNA sequence further comprises an extensive repetitive domain containing a 39 amino acid repeat unit. Southern analysis showed the repeat unit of LcKin to be variable among species, but was closely related in *L. chagasi* and *L. donovani*. Most significantly, there are high antibody titers in 98% of Brazilian VL patients to rK39, a recombinant antigen containing 6.46 copies of the 39 amino acid repeat sequence. Similar antibody levels were detected in 100% of tested Sudanese VL patients. These data indicate conservation of the repeat sequence between *L. chagasi* and *L. donovani*.

The present cloning of LcKin represents the first characterization of a gene encoding a protozoan motor protein. These microtuble based motors are involved in such varied intracellular processes as organelle and synaptic vescicle transport, chromosome segregation and spindle pole separation, nuclear fusion, protein sorting a flagellar beating. This tail domain is usually characterized by a predominantly alpha-helical structure which likely forms a coil interacting with different intracellular ligands which determine its function. The LcKin gene product is similar to members of this family in primary sequence, particularly in the putative ATP and microtubule binding domains, as well as in predicted secondary structure.

The inventive feature of LcKin was the high prevalence of antibody specific to the rK39 repeat sequence in VL patients from geographically distinct endemic regions of Brazil and the Sudan. Therefore, the rK39 repeat antigen is useful as a vaccine and as an antibody-binding antigen for a diagnostic kit for the detection and diagnosis of VL. Moreover, the inventive method for diagnosing VL using rK39 was specific for VL patients. False positives were not seen in normal patients, even normal patients from the endemic areas of Brazil and the Sudan. These data also reflect relatedness among members of the *L. donovani* complex. The data described herein provide a thorough analysis of patient antibody responses to a purified recombinant antigen (rK39) of *L. chagasi* and show a marked restriction of this response to *L. chagasi* and *L. donovani* infected patients with VL with 98% and 100% positivity in this group.. The inventive diagnostic kit and the inventive method for diagnosing VL and distinguishing VL from other infectious diseases with similar clinical presentations provides a needed tool in a clinicians hands in endemic areas of the world. Therefore, antibody reactivity to rK39 is an improved replacement for promastigote-based serological tests for the diagnosis of acute VL.

The rK39 antigen is an immunodominant B cell epitope comprising one or more copies of the Leu Glu Gln Gln Leu Arg (Asp/Glu) Ser Glu (Glu/Ala) Arg Ala Ala Glu Leu Ala Ser Gln Leu Glu (Ala/Ser) Thr (Ala/Thr) Ala Ala Lys (Met/Ser) Ser Ala Glu Gln Asp Arg Glu (Asn/Ser) Thr Arg Ala (Thr/Ala) (SEQ ID NO:3) 39 amino acid repeat sequence. Preferably the K39 antigen comprises from 1 to about 7 copies of the 39 amino acid sequence. Most preferably, the K39 antigen comprises about 6 copies of this sequence.

Gp63 is a major surface glycoprotein of *Leishmania* parasites, is highly conserved among species and is expressed in both infective and intracellular stages. The gp63 gene of *L. chagasi* has been cloned, analyzed and described in Miller et al. (*Mol. Biochem. Parasitol.* 39:276, 1990). It is significant to note that the sequence of *L. chagasi* was found to differ significantly from that published for *L. major* (Button et al. *J. Exp. Med.* 167:724, 1988). The predicted protein sequences of gp63 from *L.*

*major* and *L. chagasi* are closely related. Gp63 is a surface metalloprotease (Bouvier et al., *Mol Biochem. Parasitol.* 37:235, 1989 and Medina-Acosta et al., *Mol Biochem. Parasitol.* 37:263, 1989) that is also important for parasite entry into macrophages and survival within the phagosome. Native gp63 (ngp63) and recombinant gp63 (rgp63) elicited strong proliferative responses and IFN-γ production from leishmaniasis patients with a spectrum of clinical disease. The present invention further found the prevalence of gp63-specific antibodies among patients with clinically and geographically diverse leishmaniasis to provide a further useful diagnostic tool alone or in combination with the use of the K39 antigen for diagnosis of VL.

The prevalence of gp63-specific antibody among leishmaniasis and other disease groups was examined by an ELISA technique (described in Example 4 herein). All leishmaniasis sera were obtained from patients with active disease, and included Brazilian visceral, cutaneous or mucosal leishmaniasis from a study area in Bahia, Brazil or from biopsy-positive patients in the Sudan. Leprosy sera were from patients in Haiti where leishmaniasis has not been reported. Normal control sera were from normal volunteers in the US. Figure 6 shows that there were elevated anti-gp63 antibody levels among VL patients (mean absorbance = 0.89). In fact, 84% (42/50) of the VL patient sera tested have absorbance values greater than 3 standard deviations above the mean of normal control sera. The remaining 16% had relatively low levels of gp63-specific antibody, despite generally high titers of leishmania-specific antibody (mean absorbance value of 1.54). In contrast, cutaneous and mucosal leishmaniasis patients showed very little sero-reactivity with only two mucosal patients having absorbance values significantly above control normals. No sera samples from patients with a *T cruzi* infection or leprosy showed significantly elevated antibody levels to rgp63. These results indicate that gp63 is a potent B cell immunogen among VL patients although alone is not as good of an antigen in a diagnostic assay as is K39. However, the combination antigens of K39 and gp63 can provide a superior diagnostic kit with a reduced number of false positive results.

## EXAMPLE 1

This example illustrates the cloning of the K39 antigen. A genomic library was constructed with mechanically sheared DNA of *L. chagasi* (MHOM/BR/82/BA-2,C1) in the expression vector lambda ZAPII according to the manufacturer's protocols (Stratagene, La Jolla, CA). Recombinants were screened with serum (obtained from a patient recently treated for acute *L. donovani* infection) preadsorbed to remove anti-*E. coli* reactivity according to the procedure described in Sambrook et al. *Molecular Cloning, A Laboratory Manual, 2nd Ed.* (Cold Spring Harbor, NY, 1989).

The K39 clone was expressed to produce rK39 polypeptide and this was purified from a 25% to 40% ammonium sulfate fraction of a soluble bacterial lysate by preparative isoelectric focusing with a Bio-Rad Rotofor IEF cell and 1% 3/10 ampholytes (Bio-Rad, Richmond, CA) in the presence of 8 M urea and 10 mM dithiothreitol. Peak fractions were concentrated by a second ammonium sulfate precipitation, and dialyzed against 25 mM Tris-HCl (pH 8), 150 mM NaCl (TBS). Protein concentrations were determined using a Pierce BCA protein assay (Pierce, Rockford, IL) and purity assessed by Coomassie-blue staining following SDS-PAGE.

A radiolabeled insert of K39 was used to screen the *L. chagasi* genomic library to obtain clones containing sequences flanking the K39 gene fragment. A set of overlapping deletions of clones K39 and LcKin were generated by controlled Exonuclease III digestion (according to the procedure described in Henikoff, *Gene* 28:351, 1984) to obtain a complete sequence of both the coding and noncoding strands. Single stranded template was prepared as described in Burns et al. (*Proc. Natl. Acad. Sci. USA* 89:1239, 1992) and nucleotide sequence was obtained by the Sanger dideoxynucleotide chain termination method using $^{35}$S-labeled dATP (Sanger et al., *Proc. Natl. Acad. Sci. USA* 74:5463, 1977) or by fluorescence-based sequencing on an Applied Biosystems Sequencer Model 373A, according to the manufacturer's protocols. Sequence comparisons were made with GenPept (72.0) and Swiss-Prot (22.0) with the Lipman/Pearson method (Pearson and Lipman, *Proc, Natl. Acad. Sci. USA* 85:2444, 1988). Secondary structural predictions were made according to Garner et al. (*J. Mol. Biol.* 120:97, 1978) and Chou et al. (*Advances in Enzymology* 47:45, 1978).

DNA from *Leishmania spp.* and *T. cruzi* (MHOM/CH/00/Tulahuen C2) were isolated, digested with *Pst* I, separated by agarose gel electrophoresis, and analyzed by Southern blotting. The blots were probed with a 2.4 kb *Hind* III fragment of LcKin derived from the 5' end of the gene (probe A) or a 915 bp insert of clone K39 (probe B). Each probe was radiolabeled with $\alpha$-$^{32}$P (dCTP) to a specific activity of 9 x $10^8$ cpm/$\mu$g using random oligonucleotides as primers (Boehinger Manheim, Indianapolis). The final washes were for 1 hr in 0.1X SSC/0.5% SDS at 68 °C. Blots of *L. chagasi* DNA digested with *Bam* HI, *Hind* III and *Pst* I and no *Hind* III restriction sites were used and probed as above to assess gene copy. Probe A contained one *Bam* HI, one *Pst* I, and no *Hind* III restriction sites. Probe B did not contain sites for these restriction enzymes.

Figure 3A shows the blot of probe A and Figure 3B shows the blot of probe B. Probe A hybridized strongly to multiple *Pst* I restriction fragments of all *Leishmania spp.* tested (Figure 3A, lanes 3-9), indicating conservation in the kinesin homology domain. Polymorphism in size and number of hybridizing restriction sites were noted.

Less conservation in the repetitive domain of the LcKin gene was observed as probe B hybridized with varying intensity to *Pst* I restriction fragments of *L. chagasi* (MHOM/BR/82/BA-2,C1), *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903, obtained from Dr. Diane McMahon-Pratt, Yale University, New Haven, CT), *L. donovani* (MHOM/Et/67/HU3), *L. infantum* (IPT-1, obtained from Dr. Lee Schnur, Hebrew University-Hadassah Medical School, Jerusalem, Israel), and *L. major* (LTM p-2, obtained from Dr. David Moser, Temple Univ. Phila, PA), but not *L. guyanesis* (MHOM/BR/75/M4147) (Figure 3B, lanes 3-9). Given the intensity of the hybridization signals, the K39 repeat sequence appeared to be most closely related between *L. chagasi* and *L. donovani* (Figure 3B, lanes 3 and 7). No hybridization with either probe was observed with *T. cruzi* DNA (Figure 3, lane 10).

Using *L. chagasi* digested DNA, two *Pst* I fragments were detected with probe B, indicating the presence of a second copy of the LcKin gene or polymorphism in restriction sites present in the 3' repetitive sequences (Figure 3B, lane 3). Probe A hybridized to three fragments in each of the *Bam* HI, *Hind* III, and *Pst* I digests of *L. chagasi* DNA (Figure 3A, lanes 1-3). Taken together, the Southern blot data show that the LcKin gene is present in a minimum of 2-3 copies in the *L. chagas*i genome, and that related sequences are present in the seven species of *Leishmania* examined.

## EXAMPLE 2

This example illustrates the identification of native LcKin antigen. Rabbit anti-rK39 serum was used to probe SDS-PAGE blots of *L. chagasi* promastigote and tissue amastigote lysates to partially characterize native LcKin protein. Promastigotes were cultured in axenic media. Tissue amastigotes were obtained from spleens of Syrian hamsters or footpads of Balb/c ByJ mice and purified as described in Burns et al. (*J. Immunol.* 146:742, 1991). Rabbit anti-rK39 serum was obtained by subcutaneous immunization of an adult New Zealand white rabbit (R & R Rabbitry, Stanwood, WA) with 200 µg of purified rK39 administered in Freund's incomplete adjuvant (IFA Gibco, Grand Island, NY) together with 100 mg of N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, Calbiochem, San Diego, CA). Five weeks later, the rabbit was boosted with 200 µg rK39 in IFA alone. Four weeks later, the rabbit was boosted intravenously with 25 µg of purified rK39. The rabbit serum was collected 6 days later.

The antiserum bound specifically to purified rK39 (Figure 4A, lane 2) and to an approximately 230 kDa antigen present in amastigotes (Figure 4B, lane 6) and to a lesser degree in promastigotes (Figure 4B, lane 5). No reactivity with this serum was detected in promastigote and amastigote lysates of *L. amazonensis* (Figure 4B, lanes 7-8) indicative of the variability within this repeat. Comparable amounts of lysate were

loaded in all lanes as shown by reactivity of a rabbit anitserum raised against a constitutively expressed *L. chagasi* ribosomal phosphoprotein, Lc P0 (Skeiky et al., *J. Exp. Med.* 176:201, 1992) (Figure 4C, inset). No reactivity was apparent in pre-immune serum (Figure 4A, lane 1, Figure 4B lanes 1-4).

## EXAMPLE 3

This example illustrates the reactivity of patient sera to recognize the rK39 antigen. Patient sera were obtained from well characterized Brazilian visceral, cutaneous and mucosal patients as well as *T. cruzi* infection sera from a study area in Bahia, Brazil. African visceral and cutaneous leishmaniasis sera were from biopsy-positive patients in the Sudan. Normal sera were from clinically healthy individuals living in endemic areas of the Sudan or from the U.S. The sera were analyzed by an immunoblot assay. Immunoblots of parasite lysates or purified rK39 were prepared as described in Burns et al. (*J. Immunol.* 146:742, 1991). Filters were blocked with TBS containing 5% non-fat dried milk and probed with patient sera (1:250) or rabbit sera (1:400) diluted with TBS with 0.1% Tween-20 and 1% bovine serum albumin. Bound antibody was detected with $^{125}$I-labeled Protein A (1 x $10^6$ cpm/blot) followed by autoradiography. Both rK39 and *L. chagasi* promastigote lysates were strongly recognized by *L. chagasi* (Figure 2 A-C) and *L. donovani* (Figure 2 D-F) infection sera. Reactivity with rK39 was not observed with pools of sera obtained from mucosal (Figure 2G) or cutaneous (Figure 2H) leishmaniasis patient sera or with a pool of Chagas' disease (*e.g.*, *T. cruzi*) patient sera (Figure 2I). The pools of sera obtained from mucosal or cutaneous leishmaniasis Chagas' disease patient sera reacted strongly with promastigote lysates. These data indicate that the K39 antigen is specific to *L. chagasi* and *L. donovani* and/or K39 induces a strong antibody response only in VL patients.

## EXAMPLE 4

This example illustrates reactivity of patient sera with rK39 as determined by an ELISA. The patient sera were obtained as described in Example 3. The ELISA was conducted by diluting rK39 or *L. chagasi* promastigote lysate in coating buffer (15 mM $Na_2HCO_3$, 28 mM $NaH_2CO_3$. pH 9.6) to 1 µg/ml or 20 µg/ml, respectively. Microassay plates (Probind, Falcon, Lincoln Park, NJ) were sensitized with rK39 (50 ng) or promastigote lysate (1µg/ml) by overnight incubation at 4 °C. Plates were blocked with PBS plus 1% Tween-20 for 1 hr at room temperature. After five washes with PBS containing 0.1% Tween-20 (PBS-T), 50 µl per well of sera diluted 1:100 with PBS-T were incubated for 30 min at room temperature. The wells were again washed five times with PBS-T and bound antibody was detected by Protein A-HRP

(Zymed, So San Francisco, CA) as described in Reed et al. (*Am. J. Trop. Med. Hyg.* 43:632, 1990). Absorbance values are relative to the mean of five control sera assayed on each plate. ELISA values of at least three standard deviations greater than the mean absorbance of the normal control sera were considered positive.

I observed a high level of reactivity among VL patients with 98.2% (56 of 57) of Brazilian VL sera and 100% (52 of 52) of Sudanese VL sera exhibiting positive absorbance values. The positive absorbance values ranged from 0.05 to >2.0 (mean = 1.38) among Brazilian VL sera (Figure 5A) and from 0.094 to >2.0 (mean = 1.60) among Sudanese sera (Figure 5B). Detectable antibody to rK39 was restricted to VL patients, as little or no anti-rK39 response was observed in sera from mucosal or cutaneous leishmaniasis patients or *T. cruzi* infection sera, despite some reactivity in these latter samples with crude *L. chagasi* lysate (Figure 5).

## EXAMPLE 5

This example illustrates a method for producing and purifying recombinant gp63 (rgp63). Recombinant gp63 from *L. chagasi* and *L. donovani* was produced in *E. coli* as a non-fusion protein using T7 RNA polymerase expression system and pET plasmid expression vectors as described in Button et al. (*Mol. Biochem. Parasitol.* 44:213, 1991). Induced bacterial pellets were resuspended in lysis buffer (LB, 50 mM Tris HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA) and lysed by treatment with lysozyme and sonication. The inclusion body fraction containing rgp63 was recovered by centrifugation for 5 min at 200 x g and washed twice in LB with 4 M urea as a chaotropic agent. The final pellet containing rgp63 was solubilized in 100 mM Tris, pH 8.5, containing 8 M urea and 100 mM dithiotretiol. Following dialysis, rgp63 was isolated by ammonium sulfate fractionation, followed by preparative isoelectric focusing in the presence of 8 M urea with 3/10 ampholytes using a Rotofor IEF cell (Bio-Rad, Richmond, CA) as described in Reed et al. (*Am. J. Trop. Med. Hyg.* 44:272, 1991). Protein concentrations of rgp63 were determined using the Pierce BCA protein assay (Pierce, Rockford, IL) and purity assessed by silver-staining (Bio-Rad) after SDS-PAGE as described in Laemmli (*Nature* 227:680, 1970).

SEQUENCE LISTING

(1) GENERAL INFORMATION
(i) APPLICANT: Reed, Steven
(ii) TITLE OF INVENTION: Diagnosis of Leishmaniasis
(iii) NUMBER OF SEQUENCES: 3
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Immunex Corporation
(B) STREET: 51 University Street
(C) CITY: Seattle
(D) STATE: Washington
(E) COUNTRY: USA
(F) ZIP: 98101

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Apple System 7.1
(D) SOFTWARE: Microsoft Word, version 5.1a

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US/08/006,676
(B) FILING DATE: 15-JAN-1993

(viii) ATTORNEY INFORMATION:
(A) NAME: Perkins, Patricia Anne
(B) REGISTRATION NUMBER: 34,693
(C) REFERENCE/DOCKET NUMBER: 5004-WO

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (206) 587-0430
(B) TELEFAX: (206) 233-0644

(2) INFORMATION FOR SEQ ID NO.: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 955 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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        35                  40                  45

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    50                  55                  60

Glu Ser Met Gly Thr Ala Arg Arg Val Ala Gln Asp Phe Gln Phe
65                  70                  75
```

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80 85 90

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95 100 105

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110 115 120

Tyr Gly Gln Thr Gly Ser Gly Lys Thr Tyr Thr Met Met Gly Ala
120 125 130 135

Asp Val Ser Ala Leu Ser Gly Glu Gly Asn Gly Val Thr Pro Arg
140 145 150

Ile Cys Leu Glu Ile Phe Ala Arg Lys Ala Ser Val Glu Ala Gln
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170 175 180

Asn Glu Arg Val Ser Asp Leu Leu Gly Lys Arg Lys Lys Gly Val
185 190 195

Lys Gly Gly Gly Glu Glu Val Tyr Val Asp Val Arg Glu His Pro
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215 220 225

Ser Leu Asp Asp Val Val Arg Leu Ile Glu Ile Gly Asn Gly Val
230 235 240

Arg His Thr Ala Ser Thr Lys Met Asn Asp Arg Ser Ser Arg Ser
245 250 255

His Ala Ile Ile Met Leu Leu Leu Arg Glu Glu Arg Thr Met Thr
260 265 270

Thr Lys Ser Gly Glu Thr Ile Arg Thr Ala Gly Lys Ser Ser Arg
275 280 285

Met Asn Leu Val Asp Leu Ala Gly Ser Glu Arg Val Ala Gln Ser
290 295 300

Gln Val Glu Gly Gln Gln Phe Lys Glu Ala Thr His Ile Asn Leu
305 310 315

Ser Leu Thr Thr Leu Gly Arg Val Ile Asp Val Leu Ala Asp Met
320 325 330

Ala Thr Lys Gly Ala Lys Ala Gln Tyr Ser Val Ala Pro Phe Arg
335 340 345

Asp Ser Lys Leu Thr Phe Ile Leu Lys Asp Ser Leu Gly Gly Asn
350 355 360

Ser Lys Thr Phe Met Ile Ala Thr Val Ser Pro Ser Ala Leu Asn
365 370 375

Tyr Glu Glu Thr Leu Ser Thr Leu Arg Tyr Ala Ser Arg Ala Arg
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455                 460                 465

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470                 475                 480

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485                 490                 495

Asp Lys Met Gln Ala Leu Asn Leu Arg Leu Lys Glu Glu Gln Ala
500                 505                 510

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515                 520                 525

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530                 535                 540

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545                 550                 555

Gln Arg Glu Arg Glu Val Ala Leu Asp Ala Leu Gln Thr His Gln
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695                 700                 705

Gln Gln Leu Arg Glu Ser Glu Glu Arg Ala Ala Glu Leu Ala Ser
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                  890                 895                 900

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                  905                 910                 915

Gln Leu Glu Ala Thr Ala Ala Ala Lys Ser Ser Ala Glu Gln Asp
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(3) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3319 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) ORIGINAL SOURCE:
(A) ORGANISM: Leishmania chagasi

(ix) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(4) INFORMATION FOR SEQ ID NO. 3

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Xaa at position 7 is Asp or Glu, at position 10 is Glu or Ala, at position 21 is Ala or Ser, at position 23 is Ala or Thr, at position 27 is Met or Ser, at position 35 is Asn or Ser, and at position 39 is Thr or Ala

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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```

## Claims

I claim:

1. A method for diagnosing leishmaniasis comprising:

(a) obtaining a sample from a patient suspected of being infected with a *Leishmania* parasite, wherein the sample contains antibodies from the patient; and

(b) determining the presence of antibodies that bind to a K39 repeat unit antigen from the sample.

2. The method of claim 1 wherein the sample is a serum sample or a plasma sample.

3. The method of claim 1 wherein the K39 repeat unit antigen is one or a plurality of K39 repeat sequences, wherein the K39 repeat sequence comprises the amino acid sequence Leu Glu Gln Gln Leu Arg Xaa Ser Glu Xaa Arg Ala Ala Glu Leu Ala Ser Gln Leu Glu Xaa Thr Xaa Ala Ala Lys Xaa Ser Ala Glu Gln Asp Arg Glu Xaa Thr Arg Ala Xaa (SEQ ID NO:3), wherein Xaa at position 7 is Asp or Glu, at position 10 is Glu or Ala, at position 21 is Ala or Ser, at position 23 is Ala or Thr, at position 27 is Met or Ser, at position 35 is Asn or Ser, and at position 39 is Thr or Ala.

4. The method of claim 1, further comprises the step of determining the presence of antibodies that bind to a native or recombinant gp63 polypeptide.

5. A diagnostic kit for evaluating a patient antibody-containing sample for the presence of anti-*Leishmania* parasite antibodies, comprising a K39 repeat unit antigen.

6. The diagnostic kit of claim 5, wherein the K39 repeat unit antigen is bound to a solid phase.

7. The diagnostic kit of claim 5, wherein the kit further comprises an anti-human antibody conjugated to a detection moiety.

8. The diagnostic kit of claim 5, wherein the K39 repeat unit antigen is one or a plurality of K39 repeat sequences, wherein the K39 repeat sequence comprises the amino acid sequence Leu Glu Gln Gln Leu Arg Xaa Ser Glu Xaa Arg Ala Ala Glu Leu Ala Ser Gln Leu Glu Xaa Thr Xaa Ala Ala Lys Xaa Ser Ala Glu Gln Asp Arg Glu Xaa Thr Arg Ala Xaa (SEQ ID NO:3), wherein Xaa at position 7 is Asp or Glu, at position 10 is Glu or Ala, at position 21 is Ala or Ser, at position 23 is Ala or Thr, at position 27 is Met or Ser, at position 35 is Asn or Ser, and at position 39 is Thr or Ala.

9. The diagnostic kit of claim 5, further comprising a gp63 polypeptide, in combination with the K39 repeat unit antigen.

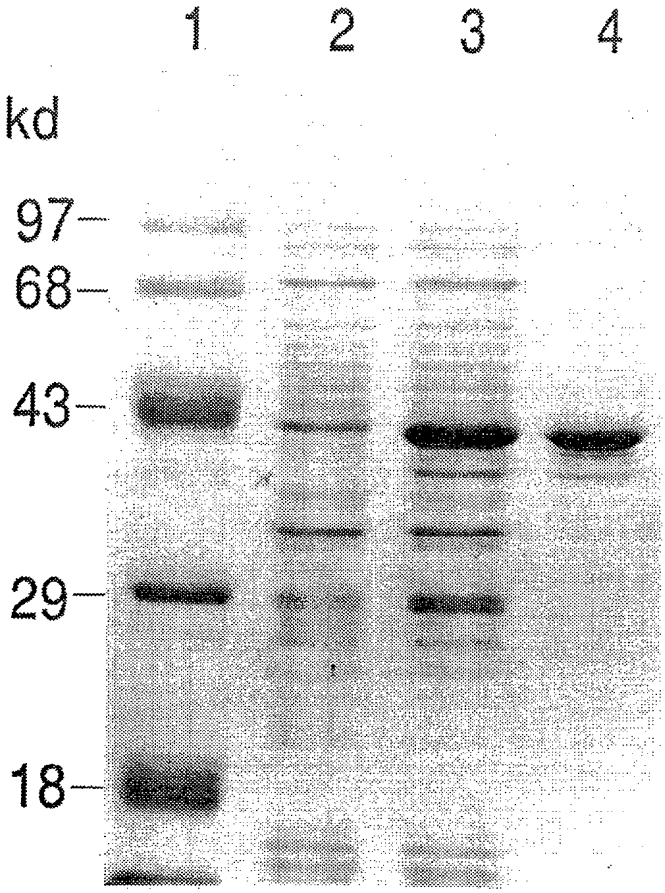



Figure 1

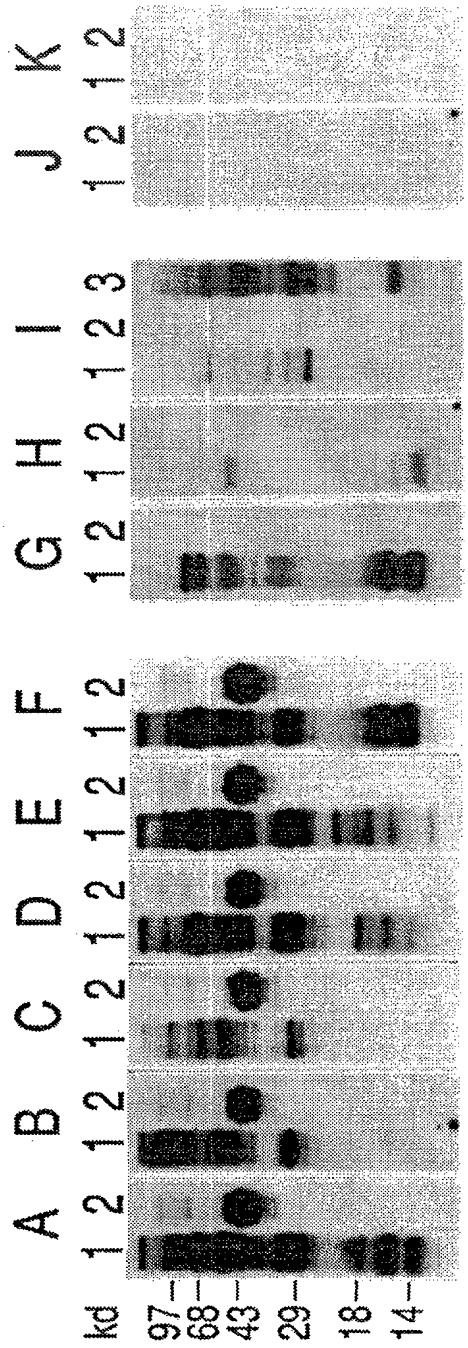



Figure 2

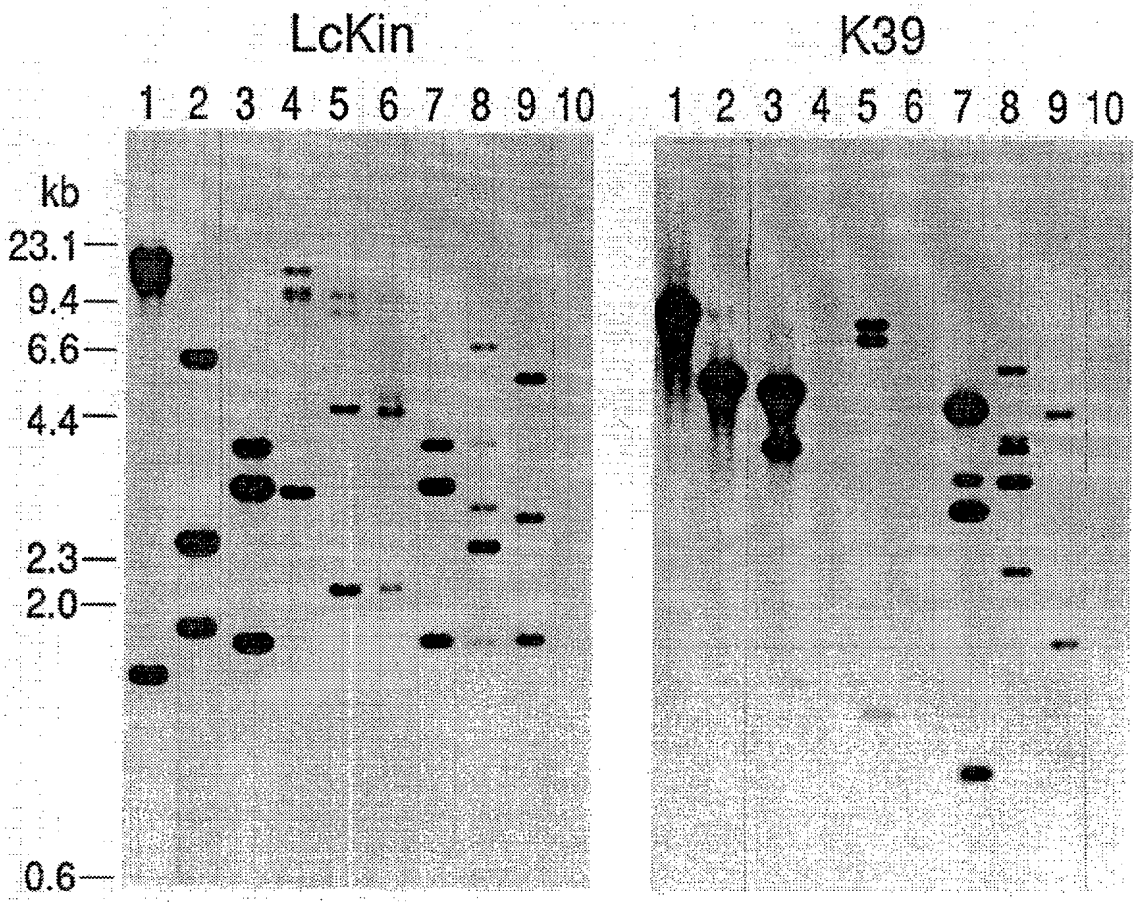



Figure 3A Figure 3B

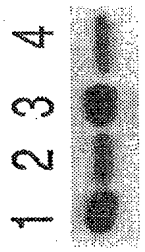



Figure 4C

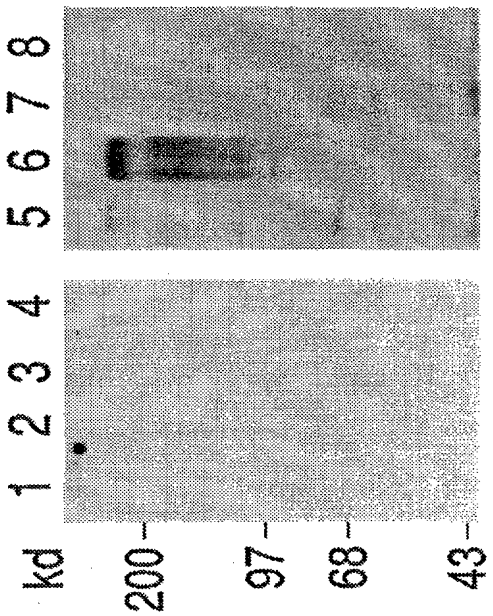



Figure 4B

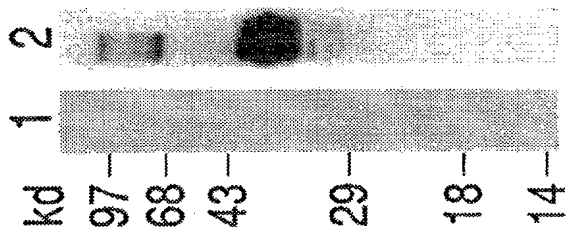



Figure 4A

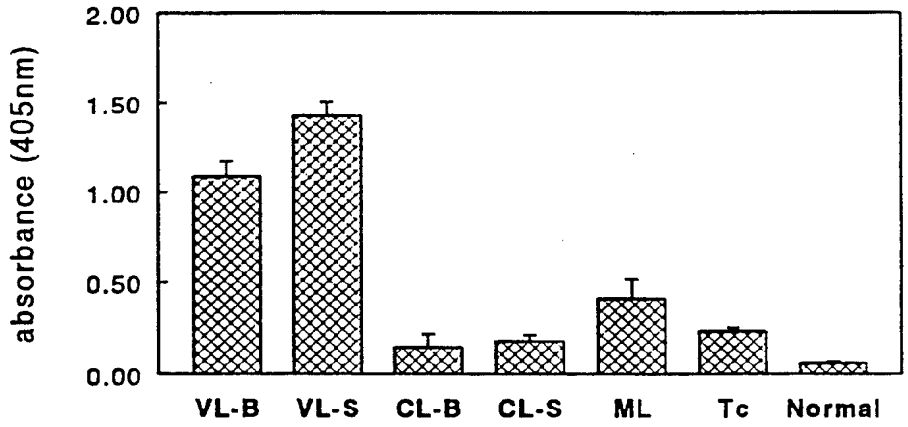



Figure 5A

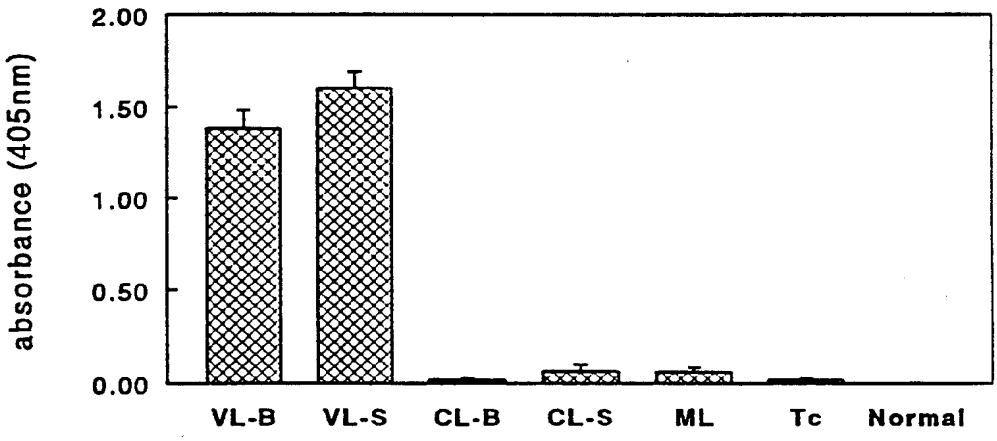
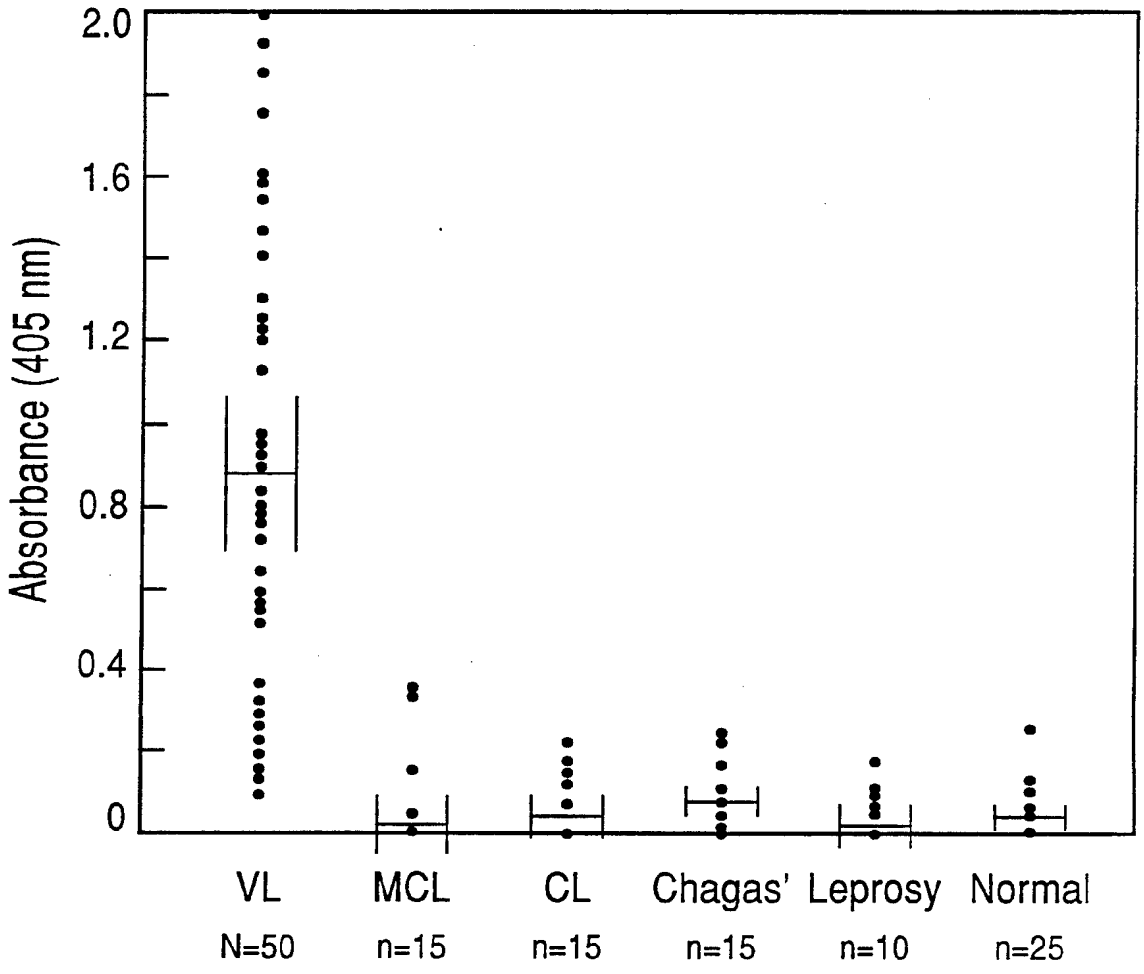



Figure 5B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1.6
1.2
0.8
0.4
0
Absorbance (405 nm)
VL
N=50
MCL
n=15
CL
n=15
Chagas'
n=15
Leprosy
n=10
Normal
n=25

Figure 6

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00324

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :G01N 33/569
US CL :435/7.22

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.22, 7.92, 7.95, 973, 975; 436/518, 528, 804, 808, 811; 530/324, 822, 810

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, IG SUITE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X,P | Proceedings of the National Academy of Sciences USA, Volume 90, No. 2, issued 15 January 1993, Burns et al, "Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis", see pages 775-779, especially page 776, paragraph bridging col. 1-2 and Fig. 3B on page 777. | 1-3, 6-8 |
| A | Journal of Clinical Microbiology, Volume 30, No. 11, issued November 1992, Zhang et al, "Use of a Recombinant 170-Kilodalton Surface Antigen of *Entamoeba histolytica* for Serodiagnosis of Amebiasis and Identification of Immunodominant Domains of the Native Molecule", pages 2788-2792. | 1-9 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search
14 MARCH 1994

Date of mailing of the international search report
APR 25 1994

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00324

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | American Journal of Tropical Medicine and Hygiene, Volume 43, No. 6, issued 1990, Reed et al , "An Improved Serodiagnostic Procedure for Visceral Leishmaniasis", pages 632-639. | 1-9 |
| A | Journal of Immunology, Volume 147, No. 10, issued 15 November 1991, Russo et al, "Human T Cell Responses to gp63, A Surface Antigen of Leishmania", pages 3575-3580. | 1-9 |